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Multiple instances of paraphyletic species and cryptic taxa revealed by mitochondrial and nuclear RAD data for *Calandrella* larks (Aves: Alaudidae)

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ABSTRACT

The avian genus *Calandrella* (larks) was recently suggested to be non-monophyletic, and was divided into two genera, of which *Calandrella* sensu stricto comprises 4–5 species in Eurasia and Africa. We analysed mitochondrial cytochrome *b* (cyt*b*) and nuclear Restriction-site Associated DNA (RAD) sequences from all species, and for cyt*b* we studied 21 of the 22 recognised subspecies, with the aim to clarify the phylogenetic relationships within the genus and to compare large-scale nuclear sequence patterns with a widely used mitochondrial marker. Cyt*b* indicated deep splits among the currently recognised species, although it failed to support the interrelationships among most of these. It also revealed unexpected deep divergences within *C. brachydactyla*, *C. blanfordi*/*C. erlangeri*, *C. cinerea*, and *C. acutirostris*. It also suggested that both *C. brachydactyla* and *C. blanfordi*, as presently circumscribed, are paraphyletic. In contrast, most of the many subspecies of *C. brachydactyla* and *C. cinerea* were unsupported by cyt*b*, although two populations of *C. cinerea* were found to be genetically distinct. The RAD data corroborated the cyt*b* tree (for the smaller number of taxa analysed) and recovered strongly supported interspecific relationships. However, coalescence analyses of the RAD data, analysed in SNAPP both with and without an outgroup, received equally strong support for two conflicting topologies. We suggest that the tree rooted with an outgroup – which is not recommended for SNAPP – is more trustworthy, and suggest that the reliability of analyses performed without any outgroup species should be thoroughly evaluated. We also demonstrate that degraded museum samples can be phylogenetically informative in RAD analyses following careful bioinformatic treatment. We note that the genus *Calandrella* is in need of taxonomic revision.

1. Introduction

The avian family Alaudidae, larks, comprises 93–97 species in 21 genera (Dickinson and Christidis, 2014; Gill and Donsker, 2014). Larks are found on all continents except Antarctica. Although the majority of the species occur in Africa, followed by Eurasia, only one species each occur in Australia and the Americas (de Juana et al., 2004; Dickinson and Christidis, 2014; Gill and Donsker, 2014). Alström et al. (2013) presented the first comprehensive phylogeny of the family, which was based on two mitochondrial and three nuclear loci for >80% of all species and representatives from all recognised genera (though not all loci were available for all species), as well as mitochondrial data for multiple subspecies. They found several non-monophyletic genera and multiple cases of unpredicted deep divergences among taxa that were treated as conspecific, as well as shallow splits between some recognised species. They concluded that "few groups of birds show the same level of disagreement between taxonomy based on morphology and phylogenetic relationships as inferred from DNA sequences", and proposed a revised generic classification.

Although the notion of cryptic species is several centuries old, reports of cryptic diversity have dramatically increased after the

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introduction of PCR methods (Bickford et al., 2007). The detection of cryptic diversity is of great importance to science as well as conservation, since the lack thereof severely can misguide conservation policy (Bickford et al., 2007; Trontelj and Fišer, 2009). Whereas cryptic diversity has been unravelled across the animal kingdom, the frequency at which it occurs varies (Trontelj and Fišer, 2009). Birds are a class offering relatively few such discoveries, and the rate of discovery of cryptic diversity is, for example, twice as high in mammals and three times higher in amphibians (Trontelj and Fišer, 2009). Most larks are cryptically coloured and patterned, and many of the species are renowned for being difficult to distinguish (de Juana et al., 2004). Surprisingly few taxonomic revisions have been undertaken using molecular and/or vocal data. Nearly all of those have proposed taxonomic splits (Alström, 1998; Ryan et al., 1998; Ryan and Bloomer, 1999; Guillaumet et al., 2005, 2006, 2008; Alström et al., 2013), except one recent paper that advocated the lumping of two species (Spottiswoode et al., 2013). As remarked by Alström et al. (2013), it seems likely that the number of presently recognised lark species is underestimated.

One of the lark genera suggested by Alström et al. (2013) to be non-monophyletic was *Calandrella*. This genus was separated into two non-sister clades: (1) *C. cinerea*, *C. brachydactyla* and *C. acutirostris* in a clade sister to the genus *Eremophila* ('horned larks'), and (2) *C. raytal*, *C. rufescens*, *C. cheleensis* and *C. athensis* in a sister position to a clade comprising the two monotypic genera *Chersophilus* and *Eremalauda*. Based on morphology, all of these relationships were totally unexpected. By priority, the name *Calandrella* was restricted to the first of these clades, while the available name *Alaudala* was resurrected for the second *Calandrella* clade. Alström et al. (2013) also found *C. brachydactyla dukhunensis* to be anciently separated from the other *C. brachydactyla* subspecies, and actually sister to *C. acutirostris* – again completely unpredicted based on morphological assessments.

The genus *Calandrella* (*sensu* Alström et al., 2013, subsequently followed by Dickinson and Christidis (2014) and Gill and Donsker (2014)) comprises four (Dickinson and Christidis, 2014) or five (Gill and Donsker, 2014) species. The taxonomy has been much debated over the years. Formerly, *C. brachydactyla* was considered conspecific with *C. cinerea* under the latter name (e.g. Meinertzhagen, 1951; Vaurie, 1959; Mayr and Greenway, 1960). Based on widely allopatric distributions, different migratory behaviour and plumage differences, Voous (1960) and Hall and Moreau (1970) proposed to treat these as separate species, and this has been followed by most subsequent authors (e.g. Glutz von Blotzheim and Bauer, 1985; Cramp, 1988; Sibley and Monroe, 1990; Keith et al., 1992). In addition, Mayr and Greenway (1960) separated *C. blanfordi* as a monotypic species; Sibley and Monroe (1990) agreed with this and also split off *C. erlangeri* (monotypic). In contrast, Dickinson and Christidis (2014) treated *C. blanfordi* as a polytypic species (with subspecies *C. b. blanfordi* in Eritrea; *C. b. daaroodensis* in northern Somalia; *C. b. eremica* in south-western Arabia; and *C. b. erlangeri* in the highlands of Ethiopia), whereas Gill and Donsker (2014) recognised *C. blanfordi* with three subspecies and *C. erlangeri* as a monotypic species. None of these authors gave any reasons for their treatments.

We here study all species and all except one of the subspecies in the genus *Calandrella* (sensu Alström et al., 2013) using full-length cytochrome *b* (cyt*b*) sequences for a sample of 46 individuals (all species but not all subspecies), a mix of full-length and short cyt*b* sequences for 114 individuals (all taxa) and Restriction-site Associated DNA (RAD) sequences for 12 individuals (all species, a few subspecies). We aim to clarify the phylogenetic relationships within the genus, to investigate the recently suggested paraphyly of *C. brachydactyla*, and to compare analyses of large-scale nuclear sequence data with a mitochondrial marker.

2. Material and methods

2.1. Study group

We analysed all of the species in the genus *Calandrella* (sensu Alström et al., 2013) as well as all of the subspecies except *C. brachydactyla orientalis* (Dickinson and Christidis, 2014; Gill and Donsker, 2014), in total 115 individuals (Table S1). Taxonomy follows Gill and Donsker (2014), except that we follow Ryan (2004) regarding the subspecies within *C. cinerea*. It should be noted that the taxonomic annotation of museum specimens is not always reliable, and we have classified the taxa according to current taxonomy (Ryan, 2004) and known distributions (Gombobaatar and Monks, 2011; BirdLife International and NatureServe, 2014; pers. obs.).

2.2. Laboratory procedures

Tissue samples were taken from toe pads of museum specimens at the Natural History Museum London (Tring; BMNH), the Royal Museum for Central Africa Tervuren (RMCA), the Natural History Museum of Denmark (ZMUC), the American Museum of Natural History (AMNH), and the University of Michigan Museum of Zoology (UMMZ).

Blood samples from live birds and tissue samples from dead embryos were extracted using standard phenol-chloroform protocol (Sambrook and Russell, 1989), whereas tissue samples from museum specimens were digested overnight at 55 °C in 100 µl lysis buffer (0.1 M Tris, 0.005 EDTA, 0.2% SDS, 0.2 M NaCl, pH 8.5) with 3–6 µl proteinase K (10 mg/ml) and then precipitated with ethanol.

We used Qiagen Multiplex PCR Kit (Qiagen Inc.), with amplification reactions containing 5 µl Qiagen Multiplex PCR Master Mix, 0.2 µl each of 10 µM forward and reverse primer (Table S2), 2 µl template DNA (5–10 ng/µl), and 2.6 µl water. We ran the PCR reactions for activation at 95 °C for 15 min; 30–45 three step cycles with denaturation at 94 °C for 30 s, annealing at varying temperatures for 90 s (Table S2), and extension at 72 °C for 90 s; final extension at 72 °C for 10 min. PCR products were checked on a 2% agarose gel, precipitated with NH_4Ac and ethanol, and then dissolved in 10–25 ml of water. We used 2 µl for sequencing of successful amplifications (BigDye sequencing kit; Applied Biosystems) in an ABI Prism 3100 capillary sequencer (Applied Biosystems).

The DNA sequence of the full cyt*b* gene was obtained from samples of wild-caught birds using amplification primers ND5-Syl (Stervander et al., 2015) and mtF-NP (Fregin et al., 2009), and internal sequencing primers Cytb_seq_H15541 (Stervander et al., 2015) and Cytb_seq_L15383 (this study; see Table S2). Owing to DNA degradation in the museum specimens, only shorter DNA fragments could be amplified, and we therefore designed specific primers based on the sequences of *C. cinerea* and *C. brachydactyla* (Table S2). For some museum samples, only a 356 basepair (bp) fragment was sequenced, and specifically to type a number of *C. acutirostris* in Kashmir, a 107 bp diagnostic fragment was sequenced. PCR success was screened on 2% agarose gels.

We performed paired-end Restriction site-Associated DNA (RAD) sequencing on fresh samples representing *C. a. acutirostris*, *C. a. tibetana*, *C. brachydactyla dukhunensis*, *C. b. longipennis*, *C. b. rubiginosa*, *C. c. cinerea*, *C. c. williamsi*, and *C. c. saturatior* from Nigeria, as well as horned lark *Eremophila alpestris* as an outgroup. Library preparation followed Baird et al. (2008) and Etter et al. (2011) with the following modifications: We considered no RNase A treatment necessary; DNA fragments were sheared with a Bioruptor Standard UCD-200 (Diagenode) and selected to a size